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Experimental Neurology 184 (2003) 839–845
Experimental Neurology
www.elsevier.com/locate/yexnr

Role of μ-opioid receptors in formalin-induced pain behavior in mice

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Received 13 March 2003; revised 3 July 2003; accepted 9 July 2003

Abstract

Intraplantar formalin injection is widely used as an experimental model of tonic pain. We investigated the role of endogenous μ-opioid receptor mechanisms in formalin-induced nocifensive behavior in mice. The flinching response induced by formalin (2%, 20 μl) was studied in mice with normal (wild type, $n = 8$) and absent (homozygous μ-opioid receptor knockout, $n = 8$) μ-opioid receptor levels. The flinch responses were counted every 5 min for 60 min post-formalin injection. Lumbar spinal cord (L4, 5) was harvested 2 h post-formalin injection to examine c-Fos expression using immunohistochemistry. The effects of naloxone (5 mg/kg, sc) administered 30 min before the intraplantar formalin injection on the flinching response of wild-type mice ($n = 7$) were also recorded. The second-phase formalin response (10–60 min after formalin) was higher in homozygous μ-opioid receptor knockout mice compared to the wild-type mice ($P < 0.01$). Naloxone administration in wild-type mice before formalin injection resulted in pain behavior similar to that observed in homozygous μ-opioid receptor knockout mice ($P > 0.05$). The c-Fos expression induced by formalin injection in the knockout mice was not different from that observed in wild-type mice. Our results suggest that the endogenous μ-opioid system is activated by intraplantar formalin injection and exerts a tonic inhibitory effect on the pain behavior. These results suggest an important modulatory role of endogenous μ-opioid receptor mechanisms in tonic pain states.

Keywords: Opioid receptors; Formalin test; c-*fos*; Naloxone; Knockout

Introduction

The formalin test is a well-established and frequently used model to study mechanisms of pain and to evaluate the analgesic action of various endogenous and exogenous substances. Unlike traditional reflex tests of nociception (e.g. tail-flick, hot-plate), pain produced by the hind paw injection of formalin results from persistent tissue damage and, thus, more closely resembles clinical pain conditions (Abbott et al., 1999; Dubuisson and Dennis, 1977; Murray et al., 1988). The intraplantar injection of a formalin solution produces a reproducible syndrome of nociceptive behaviors, which appear in two distinct phases. The first (acute) phase begins at the time of injection and lasts for about 10 min. The subsequent, second (tonic) phase starts at 10 min postinjection and has a duration of about 50 min. The first phase is thought to result from a direct chemical activation of nociceptive afferent fibers. The second phase is believed to be mediated by the activation of central sensitized neurons due to peripheral inflammation as well as ongoing activity of primary afferents (Dubner and Ren, 1999; Hunskaar and Hole, 1987; Puig and Sorkin, 1996). Using electrophysiological studies, Dickenson and Sullivan (1987) demonstrated that formalin application to a hind paw excited primary afferent C-fibers in a biphasic manner and followed a time course similar to that observed in behavioral studies. These data suggest that formalin-induced nociception is mediated by small-diameter unmyelinated nociceptors.

Studies on the role of opioid systems on nociception suggest a tonic activation of the endogenous opioids by subcutaneous formalin. Intracerebroventricular (icv) pretreatment with antiserum against β-endorphin or leu-enkephalin enhances the second-phase nociceptive responses to formalin injection. Additionally, intrathecal (it) pretreatment with antiserum against leu-enkephalin, met-enkephalin

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0014-4886/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S0014-4886(03)00346-7

or dynorphin also enhances the second-phase nociceptive responses to formalin injection (Ossipov et al., 1996; Wu et al., 2001). These studies indicate that endogenous β-endorphin and leu-enkephalin at supraspinal sites as well as leu-enkephalin, met-enkephalin and dynorphin in the spinal cord produce tonic inhibition on nociceptive response induced by formalin. Consistent with these findings, administration of an opioid receptor antagonist, naloxone, increased formalin-induced second-phase nociceptive behavior (Ossipov et al., 1996; Sugimoto et al., 1986; Wheeler-Aceto and Cowan, 1993), although some previous reports showed that naloxone had no effects (Kocher, 1988; Taylor et al., 1997) or paradoxical analgesia (Vaccarino et al., 1988) on formalin-induced nociceptive behavior. Furthermore, formalin injection elicits marked increases in β-endorphin-like immunoreactivity in ventral periaqueductal gray (PAG) and other brain regions that are important in pain control (Zangen et al., 1998). It was also demonstrated that formalin injection induces release of met-enkephalin-like substance from the rat spinal cord. Taken together, these lines of evidence suggest that the endogenous opioid system is activated by formalin and this activation results in inhibition of nociceptive responses.

The opioid receptors mediating the antinociceptive effects of endogenous opioid peptides in the formalin test are still unclear. Blocking specific opioid receptors with systemic injection of their respective antagonists, Ossipov et al. (1996) demonstrated that κ- and δ-, but not μ-, opioid receptors were involved in mediating the antinociceptive properties of the endogenous opioid system on formalin-induced nociceptive responses. In contrast, Wu et al. (2001, 2002) demonstrated that μ- and presumed ε-, but not κ- and δ-, opioid receptors in supraspinal site, as well as μ- and δ_1-, but not δ_2- and κ-, opioid receptors in spinal cord, were involved in mediating the antinociceptive properties of the endogenous opioid system on formalin-induced nociceptive responses. The reasons for these discrepancies are unclear. However, the relative specificity and selectivity of the antagonists used and the different animal species used may be contributing factors. Elucidation of the potentially different analgesic contribution of each opioid receptor subtype is of considerable importance for developing improved analgesic medications with minimal undesirable effects.

Studies in transgenic mice have been used in recent years to understand better the role of receptor mechanisms in the signaling of pain. Recently, mice lacking the μ-opioid receptor gene have been used to characterize the role of μ-opioid receptors in nociception and the analgesic actions of opioid agonists (Uhl et al., 1999). The aim of the present study was to investigate the function of endogenous μ-opioid receptor mechanisms in formalin-induced nociceptive behaviors using μ-opioid receptor knockout and wild-type mice, as well as to reexamine the effect of naloxone on the formalin-induced nociceptive behaviors in wild-type mice.

Numerous studies have shown that formalin injection in the hind paw induces rapid and transient expression of the immediate early gene c-*fos* in the spinal cord (Harris, 1998) and c-*fos* expression in the spinal cord has been widely used as a marker of neuronal activation (Dickenson et al., 1997; Munglani and Hunt, 1995). A correlation has been observed between formalin-induced nocifensive behavior and spinal Fos protein expression (Gogas et al., 1991; Hammond et al., 1992). Hence, we also investigated whether the expression of Fos protein induced by formalin injection was different in wild-type and μ-opioid receptor knockout mice.

Materials and methods

The experiments were performed using male, 8–12 weeks old, homozygous μ-opioid receptor knockout and wild-type littermates. Wild-type and homozygous μ-opioid receptor knockout mice were generated from random heterozygote crosses of mice developed in Uhl's laboratory maintaining a mixed C57/129sv background (Sora et al., 1997). The animals were maintained in a 12-h light–dark cycle and were provided with food and water ad libitum. The μ-opioid receptor knockout mice displayed no gross developmental abnormalities, were fertile, appeared healthy, and could not be distinguished from wild-type mice based on their appearance. The investigators were blinded to genotype and drug administration. The research protocol was approved by the Johns Hopkins Animal Care and Use Committee.

Formalin test

Mice were placed into a transparent observation chamber (30 × 30 × 25 cm) for adaptation 30 min before the experiments. Mice were then gently held and injected subcutaneously with 20 μl of 2% formalin solution in the plantar surface of the right hind paw with a 26-gauge needle. After formalin injection, mice were then placed into the original chamber and were observed for flinching behavior. Flinches were counted during 5-min intervals, starting from the time of formalin administration through 60 min postinjection.

Opioid antagonist study

The opioid antagonist naloxone (5 mg/kg, 250 μl) was administered subcutaneously 30 min before formalin injection in wild-type mice. Normal saline was used as the vehicle control.

Immunohistochemistry

The animals were deeply anesthetized with pentobarbital sodium (60 mg/kg, ip) and perfused with 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4) 2 h after the formalin injection. The lumbar spinal cord was removed, postfixed in the same fixative solution for 4 h, cryoprotected by immersing in 30% sucrose over-

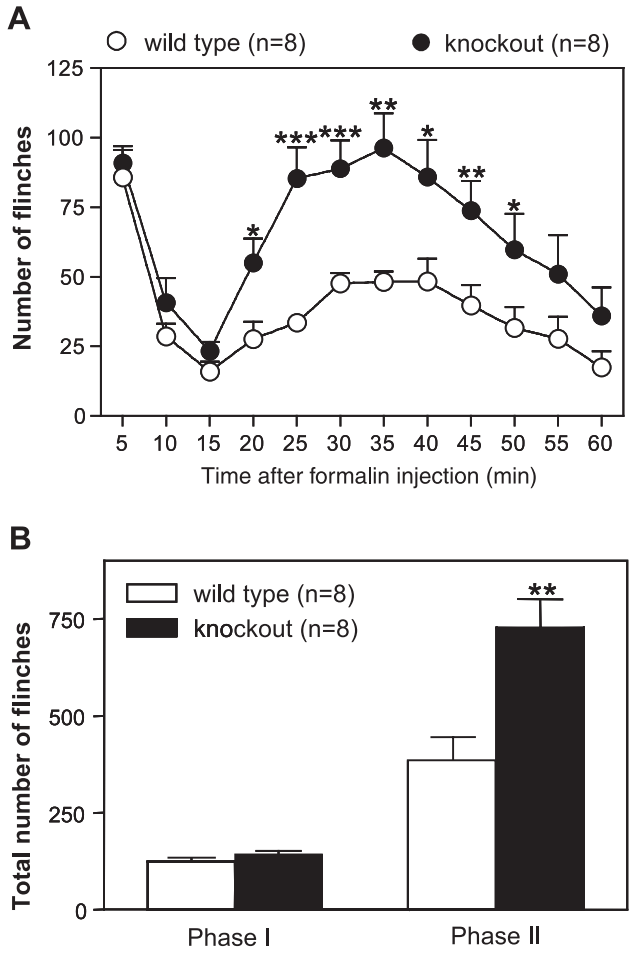

Fig. 1. Intraplantar formalin injection results in enhanced pain behavior in μ-opioid receptor knockout mice compared to wild-type mice. (A) Time course of the flinch response after formalin injection [repeated two-way ANOVA, group main effect: $F(1,14) = 9.02$, $P < 0.01$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with wild-type group]. (B) Total number of flinches during phase I (0–10 min) and phase II (10–60 min) of the nocifensive response to formalin injection ($^{**}P < 0.01$ compared with wild-type group, Student's t test).

night at 4°C and frozen-sectioned at 30 μm. Sections were incubated in polyclonal rabbit anti-Fos serum (1:4000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in 0.01 M phosphate-buffered saline (pH 7.4) containing 3% normal goat serum and 0.25% Triton X-100 for 48 h at 4°C. The sections were then incubated in biotinylated goat anti-rabbit IgG (1:200, Vector Lab) for 1 h at 37°C followed by avidin–biotin–peroxidase complex (1:100; Vector Lab) for 1 h at 37°C. The immune reaction product was visualized by catalysis of 3,3-diaminobenzidine by horseradish peroxidase in the presence of 0.01% H_2O_2.

Eight to ten sections were randomly taken from the lumbar segment (L4–L5) of the spinal cord of each mouse and were examined microscopically to quantify the Fos-like immunoreactivity (Fos-LI) positive neurons in four different areas: laminae I–II, III–IV, V–VI and VII–X.

Statistical analysis

Data are presented as the mean ± SEM. Comparisons of the number of flinching behavior or Fos-LI positive neurons between groups were performed by repeated two-way ANOVA. For comparing the total number of flinches in phase I or II formalin nociceptive behaviors between groups, Student's t test was used. Statistical significance was established at the 95% level ($P < 0.05$).

Results

μ-Opioid receptor knockout mice exhibited an increase in the number of formalin-induced flinches in the second phase

Intraplantar subcutaneous injection of 20 μl of 2% formalin produced a typical biphasic nociceptive hind paw flinch-

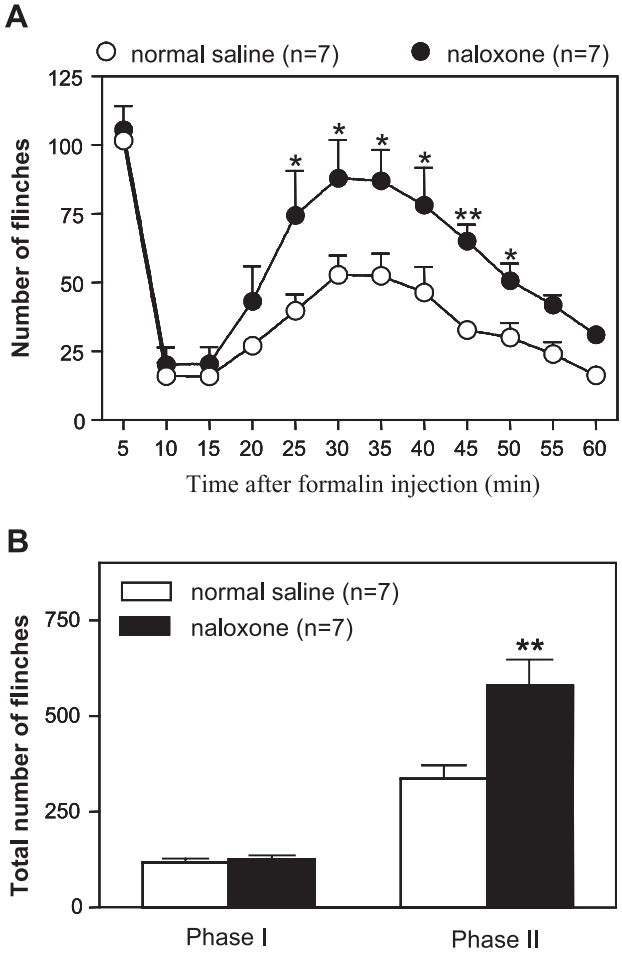

Fig. 2. Naloxone administration in wild-type mice resulted in greater phase II nociceptive responses to intraplantar injection of formalin in the hind paw. (A) Time course of the flinch response after formalin injection [repeated two-way ANOVA, group main effect: $F(1,12) = 11.29$, $P < 0.01$; $^{*}P < 0.05$; $^{**}P < 0.01$ compared with normal saline group]. (B) Total number of flinches during phase I (0–10 min) and phase II (10–60 min) of the nocifensive response to formalin injection ($^{**}P < 0.01$ compared with normal saline group, Student's t test).

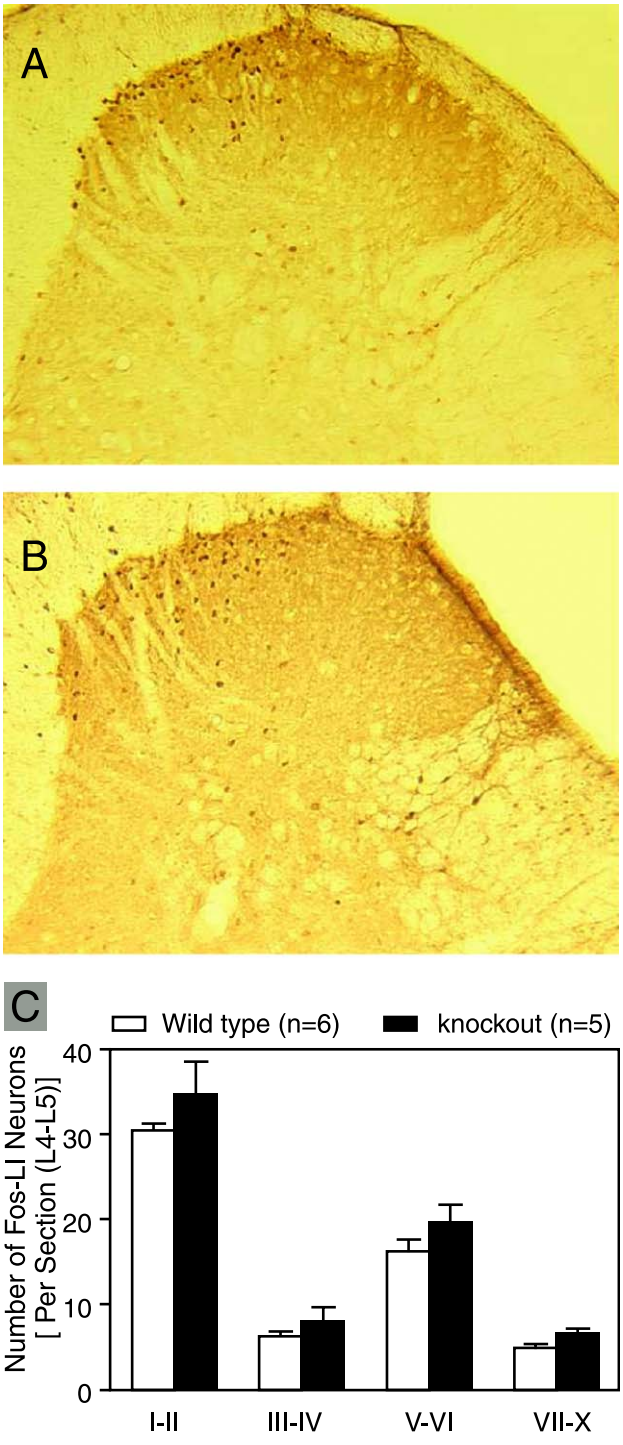

Fig. 3. Fos expression in lumbar spinal cord induced by formalin is similar in wild-type and μ-opioid receptor knockout mice. A and B are the photomicrographs of transverse sections of the lumbar spinal cord illustrating the distribution of formalin-evoked Fos-LI neurons in wild-type and μ-opioid receptor knockout mice. C shows the numbers of Fos-LI neurons in different laminae of lumbar spinal cord ipsilateral to formalin injection in wild-type and μ-opioid receptor knockout mice. The numbers of Fos-LI neurons in different laminae between wild-type and μ-opioid receptor knockout mice were not significantly different (repeated two-way ANOVA).

ing in all mice tested. The initial acute phase began immediately after injection and lasted up to 10 min. The second tonic phase began 15 min post-formalin injection, peaked at 30–35 min and dissipated 60 min after formalin administration in wild-type mice. During the acute phase, there was no significant difference in the number of flinches between μ-opioid receptor knockout mice and wild-type mice (Figs. 1A and B); however, the number of flinches during the tonic phase was significantly greater in μ-opioid receptor knockout mice than in wild-type mice [repeated two-way ANOVA, group main effect: $F(1,14) = 9.02$, $P < 0.01$ in Fig. 1A; Student's t test: $P < 0.01$ in Fig. 1B].

Naloxone increased the number of formalin-induced flinches in the second phase in wild-type mice

Compared with normal saline, subcutaneous injection of naloxone (5 mg/kg) 30 min before intraplantar formalin injection elicited a significant increase in the number of flinches observed during the phase II [repeated two-way ANOVA, group main effect: $F(1,12) = 11.29$, $P < 0.01$ in Fig. 2A; Student's t test: $P < 0.01$ in Fig. 1B] but not during the phase I formalin nociceptive behaviors in wild-type mice (Figs. 2A and B). There is no significant difference in the number of flinches observed during phase II formalin nociceptive behaviors in wild-type mice injected with naloxone (shown in Figs. 2A and B) compared with that in μ-opioid receptor knockout mice [shown in Figs. 1A and B; repeated two-way ANOVA, group main effect: $F(1,13) = 0.4$, $P > 0.05$].

No difference of formalin-induced c-Fos expression in lumbar spinal cord between wild-type and μ-opioid receptor knockout mice

Two hours after formalin injection, the lumbar 4 and 5 spinal cord was harvested to investigate the Fos expression in the spinal cord with immunohistochemistry. As previously reported (Presley et al., 1990), Fos-LI positive neurons are mostly in the laminae I–II and V–VI but less in III–IV and VII–X (Figs. 3A–C). The number of Fos-LI positive neurons in laminae I–II, III–IV, V–VI and VII–X in μ-opioid receptor knockout mice was not different compared with wild-type mice [repeated two-way ANOVA, group main effect: $F(1.9) = 2.33$, $P = 0.16$; Figs. 3A–C].

Discussion

We observed in this study that the μ-opioid receptor knockout mice developed a more robust phase II nociceptive flinching response after intraplantar injection of formalin compared with wild-type mice. In addition, we observed that naloxone administration in wild-type mice resulted in a similar increase in flinching during formalin-induced phase II response. These data suggest that endogenous opioid

peptides interacting with μ-opioid receptors exert a significant antinociceptive role during the second phase of the formalin test in wild-type mice. Our observations are consistent with that of Wu et al. (2002) who showed an increase in phase II formalin-induced licking after intracerebroventricular or intrathecal administration of the μ-opioid receptor antagonist, CTOP. Additional evidence that activation of μ-opioid receptors produces inhibition of the formalin-induced nociceptive behaviors stems from pharmacological studies. Morphine, primarily a μ-opioid receptor agonist, decreases both the acute and tonic phases of the formalin-induced nociception (Guhring et al., 2001; Shannon and Lutz, 2002; Wettstein and Grouhel, 1996). Also, the μ-opioid receptor selective agonist, DAMGO, produced inhibition of formalin-induced nociception (Gogas et al., 1991; Machelska et al., 1997; Przewlocka et al., 1999).

Martin et al. (2003) recently reported that μ-opioid receptor knockout mice showed slightly increased responses in the early but not the late phase of the formalin test. The discrepancies between our observations and that of Martin et al. may be, in part, due to methodological differences. A difference between these two studies is that 5% formalin solution was used in Martin et al.'s study, whereas a lower concentration (2%) was used in our study. A high concentration of formalin may produce a ceiling effect in the behavioral response that impedes the detection of weak analgesic effects (Tjolsen et al., 1992). Furthermore, Martin et al. (2003) measured licking behavior, whereas we measured flinching. Interestingly, systemic naloxone led to an increase in the number of flinching but a decrease in licking during the phase II formalin nociceptive responses in rats (Wheeler-Aceto and Cowan, 1993).

The endogenous opioid peptides that may be released and produce the antinociceptive effect through μ-opioid receptors during the formalin test have been studied. Subcutaneous injection of formalin elicits marked increases in β-endorphin-like immunoreactivity in the ventral PAG and other brain regions that are important in pain control (Porro et al., 1991), and also increases the release of β-endorphin in the arcuate nucleus at time points corresponding to the second phase of the formalin response (Zangen et al., 1998). Furthermore, pretreatment with an antiserum against β-endorphin enhances nociceptive response following formalin injection (Wu et al., 2001). Hamba (1988) reported that rats display increased behavioral responses to formalin injection after lesions of the arcuate nucleus of the hypothalamus, the primary site of β-endorphin-producing cells in the central nervous system. These findings indicate that formalin-induced stimulation activates the central β-endorphin system at supraspinal sites, thus inhibiting the phase II formalin-induced nociceptive responses. β-Endorphin has approximately equal affinity for both μ- and δ-receptors (Pasternak, 1993) and may also have its own unique binding site called ε-receptors (Narita and Tseng, 1998). Some effects of β-endorphin have been reported to be mediated by the stimulation of μ-opioid receptors (Monroe et al., 1996). The antinociception induced by β-endorphin microinjected into PAG of rats or injected intracerebroventricularly in mice is blocked by μ-opioid receptor antagonist CTP (D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2), indicating that μ-opioid receptors are involved in β-endorphin-induced antinociception (Monroe et al., 1996; Shook et al., 1988). So, the released β-endorphin may, at least partially, be involved in attenuation of the formalin-induced nociception through μ-opioid receptors.

Endomorphin-1 and -2 are believed to be the endogenous ligands for μ-opioid receptors with the highest affinity and selectivity for the μ-opioid receptors compared with all the other known endogenous opioids. They localize in the central nervous system regions of high μ-opioid receptor density (Zadina et al., 1999). Both intracerebroventricular and intrathecal injections of endomorphins were shown to decrease the phase I and II formalin-induced nociception (Przewlocka et al., 1999; Soignier et al., 2000). It is possible that endogenous endomorphin-1 and -2 produce antinociceptive effect via μ-opioid receptors during the formalin test. Whether there is tonic release of endomorphins after formalin injections and whether antisera against endomorphins enhance formalin-induced nociception is worthy of investigation.

We have shown that the first phase of the nociceptive response to formalin stimulation in μ-opioid receptor knockout mice is not different than wild-type mice. These findings suggest that endogenous μ-opioid receptor system is probably not tonically active under normal circumstances but is activated by formalin injection and produces its antinociceptive effects during tonic phase. This hypothesis is supported by our finding that naloxone can only enhance tonic (second)-phase formalin-induced nociceptive behavior in wild-type mice. Likewise, other investigations found that μ-opioid receptor antagonist CTOP or antiserum against β-endorphin can only enhance tonic-phase formalin-induced nociceptive behavior (Sugimoto et al., 1986; Wu et al., 2002).

μ-Opioid receptor knockout mice are similar to wild-type mice in a number of functional screening tests and biochemical observations (Sora et al., 1997). These findings suggest that the μ-opioid receptor may be independently regulated and the absence of μ-opioid receptors during development may be compatible with normal or near normal function in a number of brain circuits that express opiate receptors (Sora et al., 1997). Hence, the potential adaptive changes in other systems induced by μ-opioid receptor gene deletion could not account for the behavioral differences in nociceptive responses found in this study. To further investigate our hypothesis, we did pharmacological experiments with naloxone. Consistent with the enhanced formalin-induced tonic-phase flinch response in μ-opioid receptor knockout mice, we demonstrated that a subcutaneous injection of naloxone 30 min before formalin administration significantly enhanced the pain behavior during the tonic phase. This is consistent with the report by Ossipov et al.

(1996) who demonstrated that intraperitoneal naloxone enhanced the second-phase flinching response induced by intraplantar formalin injection in rats.

Consistent with previous report (Presley et al., 1990), our present study shows that the neurons expressing Fos after formalin injection are mainly in laminae I–II and V–VI of the dorsal horn. These laminae are sites where primary nociceptive afferent fibers terminate and also correspond to the distribution of neurons that respond to nociceptive stimuli (Bullitt, 1991; Hunt et al., 1987; Presley et al., 1990). Although our study showed that endogenous μ-opioid system may inhibit the formalin-induced flinching response, we did not observe a significant change in the expression of Fos in the spinal cord. The correlation between spinal Fos expression and nociception is controversial. Some studies suggest that there is a link between nociception and Fos expression in the spinal cord (Gogas et al., 1991; Hammond et al., 1992). However, other studies, including our present study, indicate a dissociation between pain behaviors and Fos expression (Hamalainen et al., 1996; Harris, 1998; Presley et al., 1990). The reason for this dissociation is not clear. A possibility is that Fos expression is not adequately dynamic to be a sensitive index of change in pain behavior. Most studies of Fos expression, including ours, use immunohistochemistry to count the number of positive neurons. In this technique, it is difficult to measure quantitative differences in Fos expression above the detectable level of Fos. Thus, changes in staining intensity are not taken into account. In conclusion, our study, together with the above reported studies (Hamalainen et al., 1996; Harris, 1998; Presley et al., 1990), suggests that expression of Fos in spinal neurons does not consistently correlate with pain behavior, and hence, Fos expression may not always be a reliable quantitative index of nociception.

In summary, our results suggest that the endogenous μ-opioid system is activated by a prolonged noxious stimulus, such as the intraplantar formalin injection, and exerts a tonic inhibitory effect on pain behavior. These results provide further evidence for an important modulatory role of endogenous μ-opioid receptor mechanisms in tonic pain states.

Acknowledgment

This study was supported by the NIH grant (NS26363).

References

Abbott, F.V., Ocvirk, R., Najafee, R., Franklin, K.B., 1999. Improving the efficiency of the formalin test. Pain 83, 561–569.

Bullitt, E., 1991. Somatotopy of spinal nociceptive processing. J. Comp. Neurol. 312, 279–290.

Dickenson, A.H., Sullivan, A.F., 1987. Peripheral origins and central modulation of subcutaneous formalin-induced activity of rat dorsal horn neurones. Neurosci. Lett. 83, 207–211.

Dickenson, A.H., Chapman, V., Green, G.M., 1997. The pharmacology of excitatory and inhibitory amino acid-mediated events in the transmission and modulation of pain in the spinal cord. Gen. Pharmacol. 28, 633–638.

Dubner, R., Ren, K., 1999. Endogenous mechanisms of sensory modulation. Pain (Suppl. 6), S45–S53.

Dubuisson, D., Dennis, S.G., 1977. The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. Pain 4, 161–174.

Gogas, K.R., Presley, R.W., Levine, J.D., Basbaum, A.I., 1991. The antinociceptive action of supraspinal opioids results from an increase in descending inhibitory control: correlation of nociceptive behavior and c-fos expression. Neuroscience 42, 617–628.

Guhring, H., Schuster, J., Hamza, M., Ates, M., Kotalla, C.E., Brune, K., 2001. HU-210 shows higher efficacy and potency than morphine after intrathecal administration in the mouse formalin test. Eur. J. Pharmacol. 429, 127–134.

Hamalainen, M.M., Alila, A., Johansson, G., Pertovaara, A., 1996. Cocaine-induced effects on pain behavior and C-fos expression in the spinal dorsal horn of the rat. Neurosci. Res. Commun. 19, 67–74.

Hamba, M., 1988. Effects of lesion and stimulation of rat hypothalamic arcuate nucleus on the pain system. Brain Res. Bull. 21, 757–763.

Hammond, D.L., Presley, R., Gogas, K.R., Basbaum, A.I., 1992. Morphine or U-50,488 suppresses Fos protein-like immunoreactivity in the spinal cord and nucleus tractus solitarii evoked by a noxious visceral stimulus in the rat. J. Comp. Neurol. 315, 244–253.

Harris, J.A., 1998. Using c-fos as a neural marker of pain. Brain Res. Bull. 45, 1–8.

Hunskaar, S., Hole, K., 1987. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. Pain 30, 103–114.

Hunt, S.P., Pini, A., Evan, G., 1987. Induction of c-fos-like protein in spinal cord neurons following sensory stimulation. Nature 328, 632–634.

Kocher, L., 1988. Systemic naloxone does not affect pain-related behaviour in the formalin test in rat. Physiol. Behav. 43, 265–268.

Machelska, H., Labuz, D., Przewlocki, R., Przewlocka, B., 1997. Inhibition of nitric oxide synthase enhances antinociception mediated by mu, delta and kappa opioid receptors in acute and prolonged pain in the rat spinal cord. J. Pharmacol. Exp. Ther. 282, 977–984.

Martin, M., Matifas, A., Maldonado, R., Kieffer, B.L., 2003. Acute antinociceptive responses in single and combinatorial opioid receptor knockout mice: distinct mu, delta and kappa tones. Eur. J. Neurosci. 17, 701–708.

Monroe, P.J., Hawranko, A.A., Smith, D.L., Smith, D.J., 1996. Biochemical and pharmacological characterization of multiple beta-endorphinergic antinociceptive systems in the rat periaqueductal gray. J. Pharmacol. Exp. Ther. 276, 65–73.

Munglani, R., Hunt, S.P., 1995. Proto-oncogenes: basic concepts and stimulation induced changes in the spinal cord. Prog. Brain Res. 104, 283–298.

Murray, C.W., Porreca, F., Cowan, A., 1988. Methodological refinements to the mouse paw formalin test. An animal model of tonic pain. J. Pharmacol. Methods 20, 175–186.

Narita, M., Tseng, L.F., 1998. Evidence for the existence of the beta-endorphin-sensitive "epsilon-opioid receptor" in the brain: the mechanisms of epsilon-mediated antinociception. Jpn. J. Pharmacol. 76, 233–253.

Ossipov, M.H., Kovelowski, C.J., Wheeler-Aceto, H., Cowan, A., Hunter, J.C., Lai, J., Malan Jr., T.P., Porreca, F., 1996. Opioid antagonists and antisera to endogenous opioids increase the nociceptive response to formalin: demonstration of an opioid kappa and delta inhibitory tone. J. Pharmacol. Exp. Ther. 277, 784–788.

Pasternak, G.W., 1993. Pharmacological mechanisms of opioid analgesics. Clin. Neuropharmacol. 16, 1–18.

Porro, C.A., Tassinari, G., Facchinetti, F., Panerai, A.E., Carli, G., 1991. Central beta-endorphin system involvement in the reaction to acute tonic pain. Exp. Brain Res. 83, 549–554.

Presley, R.W., Menetrey, D., Levine, J.D., Basbaum, A.I., 1990. Systemic morphine suppresses noxious stimulus-evoked Fos protein-like immunoreactivity in the rat spinal cord. J. Neurosci. 10, 323–335.

Przewlocka, B., Mika, J., Labuz, D., Toth, G., Przewlocki, R., 1999. Spinal analgesic action of endomorphins in acute, inflammatory and neuropathic pain in rats. Eur. J. Pharmacol. 367, 189–196.

Puig, S., Sorkin, L.S., 1996. Formalin-evoked activity in identified primary afferent fibers: systemic lidocaine suppresses phase-2 activity. Pain 64, 345–355.

Shannon, H.E., Lutz, E.A., 2002. Comparison of the peripheral and central effects of the opioid agonists loperamide and morphine in the formalin test in rats. Neuropharmacology 42, 253–261.

Shook, J.E., Kazmierski, W., Wire, W.S., Lemcke, P.K., Hruby, V.J., Burks, T.F., 1988. Opioid receptor selectivity of beta-endorphin in vitro and in vivo: mu, delta and epsilon receptors. J. Pharmacol. Exp. Ther. 246, 1018–1025.

Soignier, R.D., Vaccarino, A.L., Brennan, A.M., Kastin, A.J., Zadina, J.E., 2000. Analgesic effects of endomorphin-1 and endomorphin-2 in the formalin test in mice. Life Sci. 67, 907–912.

Sora, I., Takahashi, N., Funada, M., Ujike, H., Revay, R.S., Donovan, D.M., Miner, L.L., Uhl, G.R., 1997. Opiate receptor knockout mice define mu receptor roles in endogenous nociceptive responses and morphine-induced analgesia. Proc. Natl. Acad. Sci. U. S. A. 94, 1544–1549.

Sugimoto, M., Kuraishi, Y., Satoh, M., Takagi, H., 1986. Involvement of medullary opioid-peptidergic and spinal noradrenergic systems in the regulation of formalin-induced persistent pain. Neuropharmacology 25, 481–485.

Taylor, B.K., Peterson, M.A., Basbaum, A.I., 1997. Continuous intravenous infusion of naloxone does not change behavioral, cardiovascular, or inflammatory responses to subcutaneous formalin in the rat. Pain 69, 171–177.

Tjolsen, A., Berge, O.G., Hunskaar, S., Rosland, J.H., Hole, K., 1992. The formalin test: an evaluation of the method. Pain 51, 5–17.

Uhl, G.R., Sora, I., Wang, Z., 1999. The mu opiate receptor as a candidate gene for pain: polymorphisms, variations in expression, nociception, and opiate responses. Proc. Natl. Acad. Sci. U. S. A. 96, 7752–7755.

Vaccarino, A.L., Tasker, R.A., Melzack, R., 1988. Systemic administration of naloxone produces analgesia in BALB/c mice in the formalin pain test. Neurosci. Lett. 84, 103–107.

Wettstein, J.G., Grouhel, A., 1996. Opioid antagonist profile of SC norbinaltorphimine in the formalin paw assay. Pharmacol. Biochem. Behav. 53, 411–416.

Wheeler-Aceto, H., Cowan, A., 1993. Naloxone causes apparent antinociception and pronociception simultaneously in the rat paw formalin test. Eur. J. Pharmacol. 236, 193–199.

Wu, H., Hung, K., Ohsawa, M., Mizoguchi, H., Tseng, L.F., 2001. Antisera against endogenous opioids increase the nocifensive response to formalin: demonstration of inhibitory beta-endorphinergic control. Eur. J. Pharmacol. 421, 39–43.

Wu, H.E., Hung, K.C., Mizoguchi, H., Nagase, H., Tseng, L.F., 2002. Roles of endogenous opioid peptides in modulation of nocifensive response to formalin. J. Pharmacol. Exp. Ther. 300, 647–654.

Zadina, J.E., Martin-Schild, S., Gerall, A.A., Kastin, A.J., Hackler, L., Ge, L.J., Zhang, X., 1999. Endomorphins: novel endogenous mu-opiate receptor agonists in regions of high mu-opiate receptor density. Ann. N. Y. Acad. Sci. 897, 136–144.

Zangen, A., Herzberg, U., Vogel, Z., Yadid, G., 1998. Nociceptive stimulus induces release of endogenous beta-endorphin in the rat brain. Neuroscience 85, 659–662.